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PSMA-Activated Imaging Agents for Prostate Cancer

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14. ABSTRACT In preliminary studies, a potent TG analog (12ADT) was coupled to a series of pentapeptides composed of varying combinations of Asp and Glu to create PSMA-activated prodrugs. One of these prodrugs with the sequence 12ADT-Asp-Glu*Glu*Glu*Glu was efficiently hydrolyzed by PSMA and resulted in accumulation of high levels of the cleaved product in tumor tissue compared to normal tissue. The goal of this study is to take advantage of this selective accumulation of the TG analog to make a prostate cancer specific PSMA targeted imaging agent.**Specific Aims:** The specific aims of the study are: (1) To synthesize and characterize the cytotoxicity of a series of Iodide labeled Asp- or Glu-containing TG analogs. (2) To synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells. (3) To determine the *in vivo* efficacy toxicity, pharmacokinetics and biodistribution of ^{125}-I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts; (4) To evaluate added therapeutic efficacy produced by ^{131}-I labeling of the PSMA-activated prodrug in vivo against PSMA producing xenografts. **Progress:** Over the past year we have developed a 14-step synthesis to generate precursor phenolic TG analog. We documented the analogs ability to bind to the SERCA pump target. We then developed methods to couple the analog to the carrier peptide and confirmed cleavage by PSMA. Finally we developed methods to synthesize and purify the iodinated PSMA-activated agent. This compound is now under evaluation in vivo in biodistribution and imaging studies.

15. SUBJECT TERMS
PSMA, Imaging, Targeted

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Table of Contents

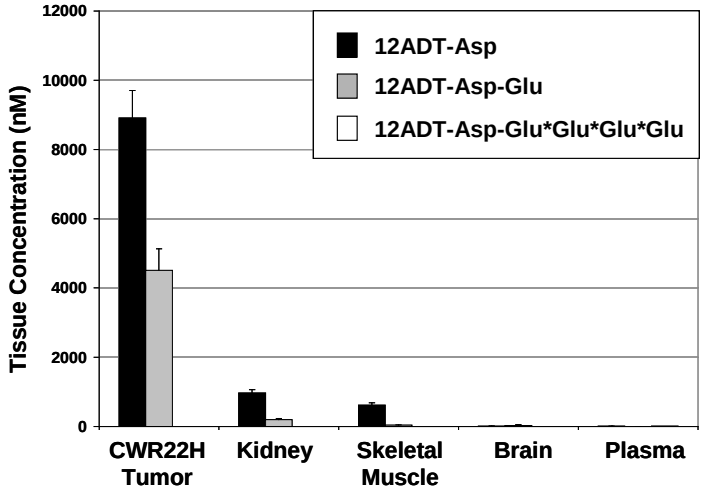
INTRODUCTION:

Our laboratory has been actively engaged in the preclinical development of methods to selectively target a highly potent cytotoxin, thapsigargin (TG), to prostate cancer cells. TG is an abundant natural product that makes up 1% of the weight of the seeds of the umbelliferous plant, *Thapsia garganica*, which grows as a weed throughout the Mediterranean basin. We have been interested in developing TG as a drug on the basis of its ability to kill both proliferating and non-proliferating prostate cancer cells at low nanomolar concentration. Previously our laboratory has demonstrated that prostate cancer cells have a remarkably low rate of proliferation (<5%/day), a finding that, in part, may explain their relatively poor response to standard antiproliferative chemotherapies. In addition, prostate cancers, like most malignancies, are a heterogeneous collection of cells that often express variable amounts of certain target proteins that are not required for cell survival. TG therapy, therefore, could overcome problems of therapeutic resistance due to low proliferative rate and heterogeneity of target expression within prostate tumors because it activates proliferation independent cell death due to its ability to inhibit the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump, a critical intracellular protein whose normal function is required by all cell types for survival. TG inhibition of the SERCA pump results in sustained elevation of intracellular calcium that leads to activation of endonucleases within the nucleus, ER stress responses and release of apoptotic factors by the mitochondria (11). TG's potent cytotoxicity, however, is not prostate cancer specific. Therefore, a strategy must be developed to target TG selectively to prostate cancer cell types while avoiding toxicity to normal, non-proliferating normal host tissues.

Our strategy for targeted TG therapy for prostate cancer has been to inactivate TG's cytotoxicity by coupling it to peptide carriers that are recognized as substrates by prostate tissue specific proteases. Since TG is a highly lipophilic compound that readily partitions into lipid membranes, coupling to a water soluble peptide carrier helps to solubilize TG while at the same time preventing it from passively entering the cell. These TG "prodrugs" can only become activated by release of the peptide by proteases present within sites of prostate cancer. Using this approach we have developed TG prodrugs that are selectively activated by prostate-specific antigen (PSA) (1) and human glandular kallikrein 2 (hK2).

In the course of developing a PSMA-activated TG therapy we identified a prodrug that was readily hydrolyzed by PSMA and selectively toxic to PSMA-producing human prostate cancers in vitro and in vivo. In the course of this work, we began to analyze tissue levels of the TG analog (12ADT-Asp) that is released from the peptide carrier by PSMA, figure 1.

A.



B.

Tissue	%ID/g
Tumor	2.68 ± 0.20
Kidney	0.17 ± 0.014
Skeletal Muscle	0.13 ± 0.15
Brain	0.0076 ± 0.008
Plasma	0.00046 ± 0.0001
Tumor/Kidney	15.4
Tumor/Skeletal Muscle	20.5
Tumor/Brain	354.3
Tumor/Plasma	5816

Figure 1. (A) Selective accumulation of 12ADT-Asp and 12ADT-Asp-Glu in tumor tissue compared to indicated normal tissue five days after single intravenous dose of 2 μmole (120 mg/kg); (B) Biodistribution of TG species (sum of 12ADT-Asp, 12ADT-Asp-Glu and 12ADT-Asp-Glu*Glu*Glu*Glu) in CWR22H tumor bearing mice. Data presented as % Initial dose (ID)/gram and tumor/tissue ratios (n=4 mice).

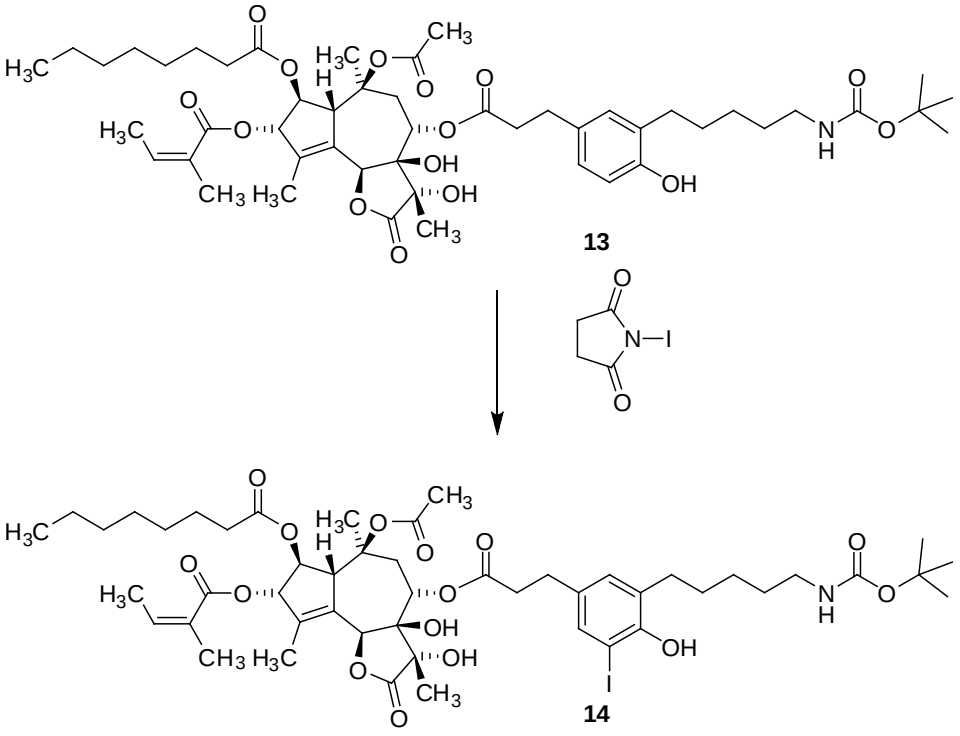
BODY:

Hypothesis: The hypothesis is that a PSMA-Activated Imaging Agent can be achieved by radiolabeling a TG analog that can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). This substrate would be readily cleaved by PSMA within prostate cancer sites resulting in the release of a radiolabeled cytotoxin (thapsigargin analog) that would selectively accumulate in prostate cancer tissue over times. This would then allow PSMA positive prostate cancers to be imaged. As a secondary objective, the cytotoxicity and antitumor efficacy of these PSMA-activated prodrugs will also be evaluated.

Specific Aims: The specific aims of the study are: (1) To synthesize and characterize the cytotoxicity and stability of a series of Iodide labeled Asp- or Glu-containing TG analogs. (2) To synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells in vitro; (3) To determine the in vivo toxicity, pharmacokinetics and biodistribution of 125-I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts.

Progress for Reporting Period 2008-2009.

In the previous progress report for this grant we described the 14 step synthesis we developed to a radiolabeled PSMA-activated prodrug. The final step in the synthesis of the radiolabeled thapsigargin analog is shown in figure 1. This compound 14 was subsequently coupled to the peptide Asp-Glu-Glu-Glu-Glu to generate the radiolabeled prodrug. On this basis, we proceeded to complete studies described in Aims 2 and 3.



Previously we also described progress on Specific Aim 2 where the goal was to synthesize iodinated PSMA prodrugs and characterize PSMA-selective activation and cytotoxicity to PSMA-producing prostate cancer cells in vitro. In initial studies we evaluated the hydrolysis of the non-iodinated PSMA prodrug (PD-14) by PSMA producing LNCaP human prostate cancer cells in vitro. In this assay we saw cleavage of the phenolic TG analog-Asp-Glu-Glu-Glu-Glu prodrug to two forms, phenolic TG (Ph12ADT) and the Asp-Ph12ADT (D-Ph12ADT) consistent with PSMA hydrolysis of the gamma linked glutamate residues, figure 2. Both analogs were observed in the conditioned media and in the cells, consistent with ability of the analogs to penetrate cell membrane once liberated from the peptide carrier. In contrast, no intact PD-14 was observed in the cell extract, consistent with the ability of the highly charged carrier peptide to keep the agent out of cells.

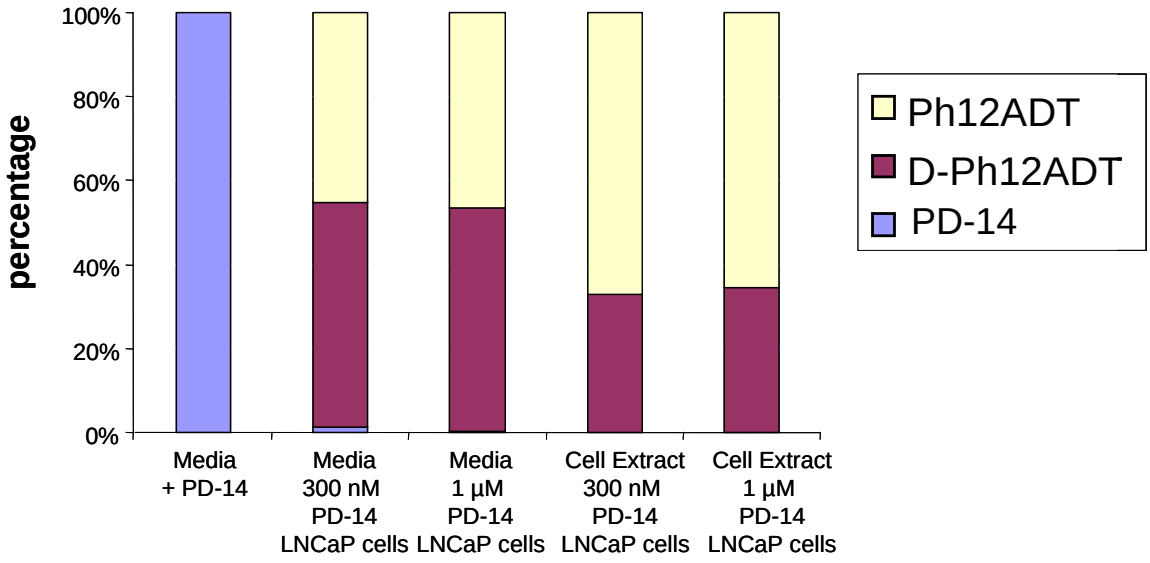


Figure 2. Relative Hydrolysis of PD-14 by LNCaP cells. LNCaP cells (confluent) were treated with media and PD-14 for 3 days. After 3 days the media was collected and the cells were scraped and extracted with acetonitrile. The media and the cell extract were run on the LCMS.

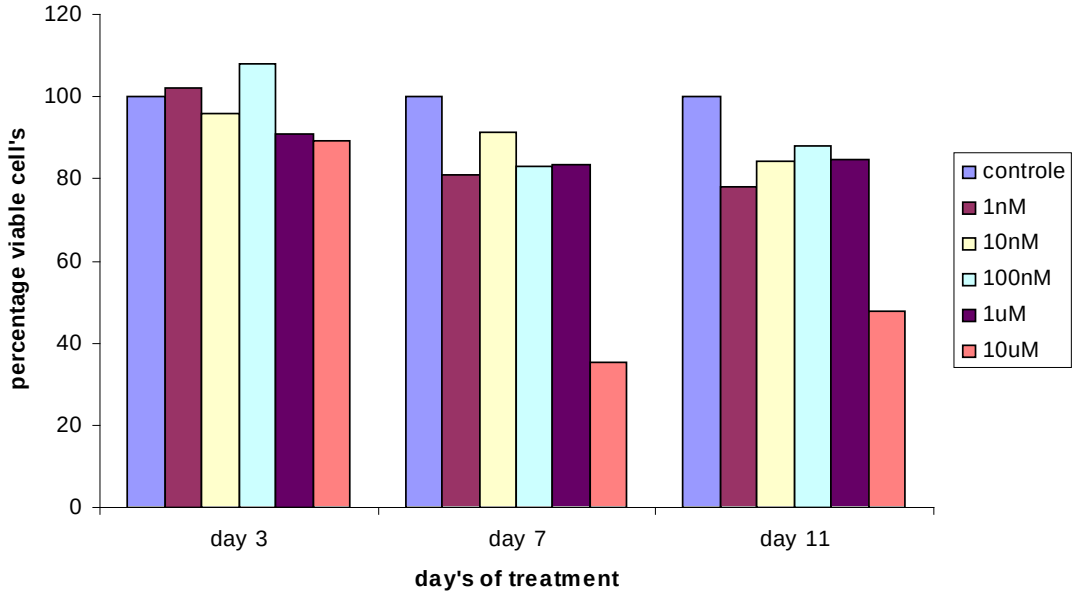


Figure 3. MTT assay evaluating varying concentrations of I-PD-14 against PSMA producing LNCaP cells.

Subsequent we developed methods to iodinate PD-14 and to purify to homogeneity. Evaluation of I-PD-14 for cytotoxicity against PSMA producing cell in tissue culture revealed toxicity of the compound at the relatively high concentration of 10 μM, figure 3. This result suggests that, while PD-14 is cleaved as evidenced by cleavage data in figure 2, insufficient TG analog is released at concentrations < 10 μM to produce a cytotoxic effect.

The goal of Specific Aim 3 was to determine the in vivo toxicity, pharmacokinetics and biodistribution of 125-I labeled PSMA-activated prodrugs in non-tumor bearing mice and mice bearing PSMA positive tumor human prostate cancer xenografts. To accomplish this goal we synthesized prodrug and established CWR22R xenografts in nude mice. In previous studies we demonstrated that CWR22R produce reasonably high levels of PSMA, although at lower levels that observed in primary human prostate cancer. However, sufficient PSMA is present in these tumors to make them suitable for these imaging studies. In contrast, these tumors make very low levels of PSA compared to human primary cancers.

In initial studies, we injected 500 μCi of the PSMA-prodrug intravenously and then sacrificed the animals 96 hrs post injection and measured total counts within the tissues, figure 4A. As a control we also injected 500 μCi of a PSA-activated radiolabeled prodrug that consists of the peptide HSSKLQ coupled to compound **14** above, figure 4B. This control was included to determine if levels of uptake in the tumor were due to PSMA cleavage or to non-specific uptake of the thapsigargin analog. The biodistribution results demonstrate that both prodrugs are predominantly cleared by the liver through the intestine. High levels of the PSMA-activated compound were observed in the PSMA-positive CWR22R xenografts, figure 4a. Lower levels were observed in the kidney, which also expresses a low level of PSMA. In contrast, for the PSA-activated compound, minimal activity was observed in tumor tissue compared to most other organs, figure 4b. These results suggest that tumor uptake is due to PSMA cleavage of the prodrug to released the radiolabeled thapsigargin analog, which then accumulates in tumor tissue.

A. **B.**

Figure 4. Uptake of (A) PSMA-activated prodrug or (B) PSA-activated prodrug. Counts were determined on whole organs using gamma-counter (Beckman-Coulter) at 96 hours post injection of compounds. Counts/gram are shown for representative animals.

Based on these biodistribution data we proceeded to evaluate the PSMA-activated prodrug as an imaging agent. In these experiments, we utilized the human prostate cancer cell line PC-3. This line is androgen receptor negative and does not produce PSMA. We transfected this line with a PSMA vector to generate PSMA producing PC-3 tumors. We then inoculated the PSMA negative PC-3 cells in one flank of the animal and the PSMA-positive PC-3 cells in the opposite flank, figure 5. SPECT/CT imaging was performed at the indicated

time points. These studies demonstrate no imageable uptake up to 15 hrs post injection with significant uptake in the heart and liver consistent with prodrug in the circulation. Beginning at 24 hrs PC-3PSMA positive tumor uptake is observed along with considerable signal from the liver and intestines. By 48 hrs, tumor uptake is more significant, although some non-specific uptake into the PSMA negative tumor is observed, figure 5. Less signal is seen within the liver, consistent with ongoing clearance through the bowel. By 72 hours the signal is diminished in the PSMA positive xenograft but much lower to no signal is seen in the PSMA negative tumor, figure 5.

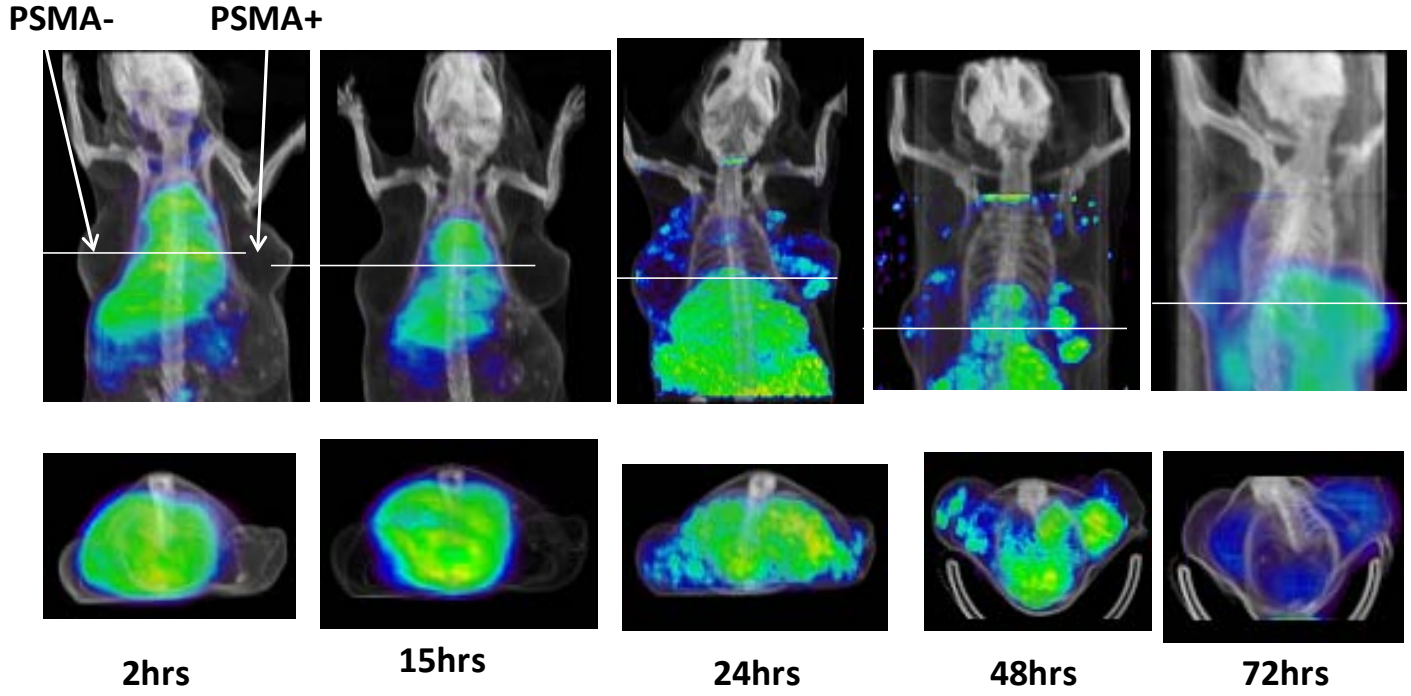


Figure 5. SPECT/CT Imaging of PSMA thapsigargin pro-drug with 125-I as a radio tracer. Top image is a 3D-reconstruction image, the bottom image is the corresponding (white line) transverse section. On the left flank of animals is a the PC3-PSMA tumor (PSMS producing), on the right side a PC3-vector control (PSMA negative) tumor as a negative control. Specific tumor uptake in the PSMA positive tumor is noted >24hrs.

Key Research Accomplishments

- Developed a 14 step method to synthesize a radioiodinateable TG analog
- Developed methods to synthesize a PSMA-cleavable prodrug
- Synthesized 25 mg of PSMA prodrug precursor for iodination
- Demonstrated cleavage of PSMA prodrug by PSMA producing prostate cancer cells
- Documented cytotoxicity of the PSMA prodrug
- Developed methods to synthesize and purify 125-I labeled PSMA prodrug
- Completed in vivo studies on distribution of the 125-I labeled prodrug
- Performed initial imaging studies demonstrating tumor uptake of the 125-I labeled compounds

Reportable Outcomes

- "PSMA-Activated Imaging Agents" Presented at Tri-Institutional SPORE meeting Newport, RI, 2008 Podium Presentation:
- "Targeted imaging of prostate cancer: smart-bomb pro-drug mechanism" JP Michiel Sedelaar, John T Isaacs, Samuel R Denmeade, Moderated Poster Session European Urological Association Annual Meeting, Stockholm, Sweden, 2009.
- "Targeted imaging for prostate cancer: the smart-bomb pro-drug mechanism", JP Michiel Sedelaar, John T Isaacs, Samuel R Denmeade, Poster Presentation American Association for Cancer Research Annual Meeting, Denver CO, 2009
- Training: Provided support for training program of Post-Doctoral Fellow J. Michiel Sedelaar

Conclusion

The goal of these studies is to develop a PSMA-Activated Imaging Agent by radiolabeling a TG analog that can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). This substrate would be readily cleaved by PSMA within prostate cancer sites resulting in the release of a radiolabeled cytotoxin (thapsigargin analog) that would selectively accumulate in prostate cancer tissue over times allowing PSMA positive prostate cancers to be imaged. Over the first year of funding our major accomplishment was to develop a synthetic method to generate phenolic ring containing TG analogs that can be readily iodinated. We also developed methods to rapidly radiolabel and purify the compound. Having achieved these goals, over this year of funding we have completed studies to assess the biodistribution and imaging potential of a PSMA-activated 125-I labeled compound. We demonstrate that the compound is cleared through the liver and has considerable tumor uptake compared to other normal organs. Over the ensuing year of funding we will work towards optimizing this approach by evaluating alternative analogs to evaluate whether we can get enhanced tumor uptake compared to normal tissues.

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